AD_____

Award Number: W81XWH-12-1-0302

TITLE: White matter glia pathology in autism

PRINCIPAL INVESTIGATOR: Gregory A. Ordway, Ph.D.

CONTRACTING ORGANIZATION: East Tennessee State University
 Johnson City, TN 37614-6500

REPORT DATE: September 2013

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                           Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2013	Annual	01 September 2012 – 31 August 2013

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
White matter glia pathology in autism	W81XWH-12-1-0302

5b. GRANT NUMBER
W81XWH-12-1-0302

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Gregory A. Ordway, Ph.D.

Michelle J. Chandley, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

E-Mail: Ordway@etsu.edu and chandlem@etsu.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
East Tennessee State University
807 University Pkwy
Johnson City, TN 37614-6500

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overall goal of this proposed work is to begin elucidating the cellular and molecular basis of white matter abnormalities in autism spectrum disorder (ASD). Using laser capture microdissection (LCM), subpopulations of white matter glia, i.e. astrocytes and oligodendrocytes, can be isolated independently of other brain cells and analyzed for gene expression differences that might contribute to ASD pathology. The combination of LCM and transcriptional analysis offer an innovative approach to determining the cellular basis of brain pathology in ASD. Due to the time intensive nature of LCM, the first year of the grant was set aside primarily for sample collection. During this year, multiple LCM instrument failures have slowed the progress of cell capture. Despite setbacks, we were able to conduct preliminary feasibility studies and precisely determine the course of action to complete the project in the second year of the award. Our university has purchased a new Arcturus XT LCM instrument that is expected to arrive and be functional within the first two weeks of October this year. This annual report summarizes the efforts made to overcome unforeseen obstacles that have modestly impeded the progress of this grant.

15. SUBJECT TERMS
Laser Capture Microdissection, RNA Integrity Number (RIN), Transcriptional Analysis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	9	

Table of Contents

Introduction

White matter abnormalities in the brains of autism spectrum disorder (ASD) patients have been heavily documented. Most of these data have been produced using imaging techniques that reveal decreased structural integrity within the white matter of ASD brains[1–5]. It is still unknown what mechanism is responsible for this gross anatomical pathology. In the past, ASD has been considered a dysfunction of neurons and their connections. However based on findings from our lab and others, the most abundant white matter cell type, glia, could contribute to these white matter abnormalities. Glia cells provide functional support to neurons, facilitating electrical conduction along neuronal axons and promoting general neuronal health. Due to these essential functions of glial cells in neuronal function, it is feasible that glia could contribute, either primarily or secondarily, to ASD pathology. There are reported changes in white matter glial cell structure and cell marker expression in ASD[6–8]. This project focuses on examining ASD white matter pathology at a cellular level. A combination of laser capture microdissection (LCM) and global transcriptional analysis will be used to study both white matter astrocyte and oligodendrocyte populations in the anterior cingulate cortex, the two principal cell types in white matter. Gene expression differences within these cell populations will be compared between ASD and developmentally normal control brains in order to elucidate a molecular and cellular basis of white matter pathology in ASD.

Body

After receiving this award, the Arcturus Veritas LCM instrument that was to be used for cell capture became nonfunctional. This machine works based on a technology that adheres tissue to a polymer-coated cap using an infrared laser. If the machine loses the ability to correctly place the cap or fire the laser, sample capture is not possible. Both of these instrument errors occurred. Since the instrument was still under a service contract, the company made several attempts to repair the instrument including a complete overhaul of the system. Based on lack of service support for an instrument that is no longer sold, the company was unable to restore the machine to working order. The determination that the Arcturus Veritas machine could not be repaired was made several months following the awarding of this grant.

In efforts to reduce downtime, we immediately started the process of purchasing a new LCM instrument. Based on available purchasing funds, we wished to move away from LCM cap-based technology because of the added expense of consumables for this method of LCM. Our university selected the Leica LMD6500 instrument for purchase, primarily based on their claims that this instrument could be used for downstream gene expression research. Leica assured us that the instrument was superior and capable of providing quality RNA following cell capture. Following purchasing and delivery delays, the LMD6500 instrument was installed on May 8, 2013. Once the instrument was installed, we immediately starting performing validation experiments to determine the suitability of this instrument for RNA analysis. Between May and September, we worked closely with the company to get the machine in full working order. There were several pieces of equipment on the new instrument that had to be replaced, which resulted in multiple service visits to repair the machine. The new software system on the instrument also posed problems thus hindering the ability to capture samples. The machine was nonfunctional for roughly half of this five-month period. The machine is still experiencing both hardware and software problems.

However during this time, we were able to perform enough experiments to determine if the LMD6500 instrument was capable of producing captured samples with high RNA integrity. RNA was isolated from captured tissue samples and analyzed using the Bioanalyzer Pico 6000 RNA chips (Agilent Technology) to determine both RNA quality and quantity. Acceptable RNA quality is defined as a RNA Integrity Number (RIN) of 5.0 or greater. This is the minimum requirement for our downstream RNA amplification process, and it is imperative that this RIN threshold is met. Due to the limited amount of sample that can be obtained from LCM, RNA amplification is an unavoidable preparation step in the transcriptional analysis outline in this grant. As a result, RIN quality must be as high as possible to reduce the likelihood of experimental artifacts in downstream applications from possibly degraded LCM samples. Studies show that RNA degradation can alter results by producing false changes in gene expression analysis[9–11]. To date, we have been unable to reliably produce captured samples with acceptable RNA quality using the LMD6500 instrument. In order to rule out RNA degradation due to tissue and sample preparation, studies were performed using different slides types, collection methods, tissue types, staining protocols, laser firing approaches, and RNA isolation kits. Based on our results, these preparation steps produced no difference in the RIN obtained from samples captured using the Leica LMD6500 instrument. In fact, after detailed analysis, we concluded that this instrument destroys RNA quality and that the use of the instrument's UV laser and its movement is the causal factor.

Arrangements were then made to complete the grant using the facilities and equipment at David H. Murdock Research Institute (DHMRI) in Kannapolis, NC. The DHMRI employs experts in the field of laser capture microdissection and transcriptional analysis. This research facility has a Zeiss Palm Microbeam laser capture instrument as well as all the resources need to complete the transcriptional analysis outlined in this grant. DHMRI made the Palm instrument available to us on a fee-for-service basis. We considered this a possible alternative because the UV laser on the Palm system is lower energy and the laser cutting movement is guided by a different engineering technology than the UV beam on the Leica system. Work began immediately to validate the Zeiss Palm LCM system for RNA analysis. Various samples were collected to test the quality of post-capture samples. After analyzing the samples using the Agilent Bioanalyzer, it was concluded that a RIN of 5.0 or greater could not be achieved using this laser capture technology.

Based on the data produced from the Leica LMD6500 and Zeiss Palm Microbeam instruments, we concluded that instruments that focus the UV cutting laser through the microscope objective will not produce reliably high quality RNA. The reasons why are not fully understood, but it is possible that microscopic irregularities within the objective lens of these instruments may disperse the UV radiation and that would result in RNA damage. Based on this conclusion, our university has ordered an Arcturus XT LCM instrument. This instrument is an updated version of the Veritas model that we have utilized in the past to generate all the LCM data published from our lab[12,13]. Again, this instrument uses an infrared laser and we have previously confirmed that this method of cell capture does not damage RNA quality. Hence, despite our attempts to move away from cap-based LCM technology because of the cost of consumables needed for this capture method, we have determined Arcturus instruments are the only instruments that can provide reliable RNA quality using the LCM methods outlined in our Statement of Work. We have used instruments from this company, including the XT model, for over five years to produce multiple publications. Other researchers at our university have also utilized this machine to conduct microarray and next generation sequencing studies on frozen human tissue. Based on our past history with this brand of instrument, we feel positive that the work outlined in this proposal can be completed in a timely fashion once we have access to this instrument (anticipated to be in the first two weeks of October).

Despite delays in laser capture, we were able to use that time to ensure that sample preparation steps such as tissue staining and RNA isolation provided adequate amounts of high quality RNA for downstream applications. These experiments were necessary since high RNA integrity is of the upmost importance for the analysis outline in this grant. Most of these experiments went into validating our RNA amplification protocol. The RNA amplification step in sample preparation is a sensitive process that could introduce experimental artifact if not performed correctly or tailored to specific biological samples. Experiments were performed using both commercially available kits and LCM specialized protocols to determine the most effective and reliable method for RNA amplification. The first strand synthesis step of RNA amplification is crucial since it creates the basic template for amplification. Most methods are based on a 3' bias selection for this step of amplification. This selection technique is beneficial for reducing other RNA species such as rRNA and tRNA from the sample prior to mass amplification. This selection reduces potential bias toward more abundant RNAs since mRNA and non-coding RNA are significantly outnumbered by other RNA species in the samples. For sample collected from LCM, this 3' bias could reduce fidelity in amplification. Using frozen human tissue for these studies means that less that optimal RNA quality is never achieved because of decay that occurs during the brain collection process. A 3' bias selection could inadvertently exclude mRNAs that are susceptible to 3' degradation. Unlike other kits available on the market, the NuGEN amplification kits create a first strand using 3' and random primers giving better transcriptome coverage and reducing potential bias. This feature makes the kits ideal for LCM samples based on its tolerance for less than optimal RNA integrity and a small RNA input requirement of 500 picograms. These kits provide robust amplification typically resulting in microgram amounts of RNA from very little template input. After the completion of NuGEN's innovative short protocol that requires very little hands-on time, enough RNA is produced to use in any downstream analysis.

Key Research Accomplishments

- We performed validation tests on multiple LCM instruments to ensure the machine could produce reliable data. This is performed to assure successful capture of specific cells and to demonstrate that high RNA quality could be extracted. We concluded that the Arcturus LCM technology with infrared laser capture is the only method that can be used to ensure sample integrity following the LCM protocol we have outline in this grant. This instrument has previously been tested and validated by our lab for its ability to produce sample of high RNA integrity for downstream analysis.

- We performed optimization experiments to ensure that sample preparation protocols supported RNA work by maintaining sample integrity during sample processing.
- We tested multiple RNA amplification methods to determine the most suitable for LCM sample preparation. Results conclude that the commercially available NuGEN amplification kits are the most reliable for producing amplified RNA with high fidelity to the original starting sample.

Reportable Outcomes

Based on the timeline established for this grant, no reportable outcomes were expected in the first year. Due to the time intensive method of collecting samples using LCM technology, the approved statement of work outlined the first year to be predominately dedicated to cell capture. Reportable data from transcriptional analysis was not expected until year two.

Conclusion

This annual report outlines the problems we have faced in using laser capture to perform the work proposed in this grant. This instrument is an essential cornerstone of this project, and we have gone to great pains to make sure we the LCM instrument can produce reliable data based on high quality of isolated RNA. While validating the machines mentioned above, detailed experiments were also carried out to ensure that all experimental protocols were sufficiently optimized so there would be no further delays in completing this work. A new Arcturus XT laser capture instrument that uses infrared laser technology has been purchased and will be installed at our university within the first two weeks of October. Because we have already validated this instrument for RNA work, sample collection can begin immediately upon its arrival. Since we are familiar with the technology and are already set up for capture on this machine, there will be no further delay. We feel confident about our abilities to complete the proposed work in the time period remaining and we will do so by intensifying our efforts in the year ahead to make up for mechanical/technical setbacks in the first year.

References

1.   Noriuchi M, Kikuchi Y, Yoshiura T, Kira R, Shigeto H, Hara T, et al. Altered white matter fractional anisotropy and social impairment in children with autism spectrum disorder. Brain research [Internet]. 2010 Nov 29 [cited 2013 Mar 11];1362:141–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20858472

2.   Ingalhalikar M, Parker D, Bloy L, Roberts TPL, Verma R. Diffusion based abnormality markers of pathology: Toward learned diagnostic prediction of ASD. NeuroImage [Internet]. 2011 May 14 [cited 2011 May 27]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/21609768

3.   Groen WB, Buitelaar JK, van der Gaag RJ, Zwiers MP. Pervasive microstructural abnormalities in autism: a DTI study. Journal of psychiatry & neuroscience : JPN [Internet]. 2011 Jan [cited 2011 Jan 21];36(1):32–40. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3004973&tool=pmcentrez&rendertype=abstract

4.   Radua J, Via E, Catani M, Mataix-Cols D. Voxel-based meta-analysis of regional white-matter volume differences in autism spectrum disorder versus healthy controls. Psychological medicine [Internet]. 2010 Nov 16 [cited 2011 Jan 21];1–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21078227

5.   Sundaram SK, Kumar A, Makki MI, Behen ME, Chugani HT, Chugani DC. Diffusion tensor imaging of frontal lobe in autism spectrum disorder. Cerebral cortex (New York, NY : 1991) [Internet]. 2008 Nov [cited 2011 Jun 20];18(11):2659–65. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2567426&tool=pmcentrez&rendertype=abstract

6.   Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo C a. Neuroglial activation and neuroinflammation in the brain of patients with autism. Annals of neurology [Internet]. 2005 Jan [cited 2010 Sep 28];57(1):67–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15546155

7.   Laurence JA, Fatemi SH. Glial fibrillary acidic protein is elevated in superior frontal, parietal and cerebellar cortices of autistic subjects. Cerebellum (London, England) [Internet]. 2005 Jan [cited 2013 Mar 5];4(3):206–10. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16147953

8.   Gozzi M, Nielson DM, Lenroot RK, Ostuni JL, Luckenbaugh DA, Thurm AE, et al. A magnetization transfer imaging study of corpus callosum myelination in young children with autism. Biological psychiatry [Internet]. 2012 Aug 1 [cited 2013 Apr 8];72(3):215–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22386453

9.   Auer H, Lyianarachchi S, Newsom D, Klisovic MI, Marcucci G, Marcucci U, et al. Chipping away at the chip bias: RNA degradation in microarray analysis. Nature genetics [Internet]. 2003 Dec [cited 2013 Sep 6];35(4):292–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14647279

10. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC molecular biology [Internet]. 2006 Jan [cited 2013 Aug 6];7:3. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1413964&tool=pmcentrez&rendertype=abstract

11. Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. Journal of biomolecular techniques : JBT [Internet]. 2004 Sep [cited 2013 Sep 9];15(3):155–66. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2291693&tool=pmcentrez&rendertype=abstract

12. Chandley MJ, Szebeni K, Szebeni A, Crawford J, Stockmeier CA, Turecki G, et al. Gene expression deficits in pontine locus coeruleus astrocytes in men with major depressive disorder. Journal of psychiatry neuroscience JPN. 2012;38:120110.

13. Ordway GA, Szebeni A, Chandley MJ, Stockmeier CA, Xiang L, Newton SS, et al. Low gene expression of bone morphogenetic protein 7 in brainstem astrocytes in major depression. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP) [Internet]. 2011 Sep 6 [cited 2011 Sep 9];1–14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21896235

Appendices

None attached

Supporting Data

None attached